

REMARKS***Claim Amendments***

Method claims 1 and 2 have been cancelled in an effort to expedite prosecution of this application to allowance, for reasons discussed further below.

Method claims 3 and 4 have been amended to more specifically define the cancer being treated as being “selected from colon, breast, prostate, lung and skin,” specification support for which if found, most particularly, at page 14, lines 25-29.

Method claims 5 and 6 have been cancelled in an effort to expedite prosecution of this application to allowance, for reasons discussed further below.

Claims 7 and 8 remain as originally filed.

Claims 9-14 have been cancelled as being in a “use” format not generally accepted under U.S. practice.

The above amendments are being made without waiver or prejudice to Applicant’s right to prosecute any deleted subject matter in one or more continuing applications. Following entry of the above amendments, claims 3-4 and 7-8 remain pending in this application.

Rejection and Objection to Specification and Claims Based on Spelling

At page 2 of the Action the Examiner has objected to the specification and claims for allegedly not complying with 35 U.S.C. § 112, as not being written in “full, clear, concise and exact” in that *British* English spelling is consistently used throughout rather than *American* English spelling. This objection is respectfully traversed. As specifically recognized by §608.01 of the MPEP, 37 CFR 1.52(b)(1)(ii) only requires the application to be in the English language, and there is no additional requirement that the English must be American English:

Examiners should not object to the specification and/or claims in patent applications merely because applicants are using British English spellings (e.g., colour) rather than American English spellings. It is not necessary to replace the British English spellings with the equivalent American English spellings in the U.S. patent applications. Note that 37 CFR 1.52(b)(1)(ii) only requires the application to be in the English language. There is no additional requirement that the English must be American English.

(MPEP §608.01, 8th Edition, Rev. 6, August 2007).

It is therefore respectfully requested that these objections and/or rejections based on the British English spelling be withdrawn.

Claim Rejections - 35 USC § 112, 1st Paragraph

Claims 3-6 are rejected under 35 U.S.C. 112, first paragraph, “because the specification, while being enabling for treating colorectal cancer, does not reasonably provide enablement for ‘treating cancer.’” While Applicant respectfully disagrees with the Examiner’s statement, the above amendments more specifically direct the method of treatment claims toward particular cancers that are highlighted in the specification. Thus, in order to expedite the prosecution of this application to allowance, claims 1 and 2 (directed toward the production of an antiangiogenic and/or vascular permeability reducing effect) and claims 5 and 6 (directed toward a method for the treatment of a cancer involving a solid tumour) have been cancelled *without prejudice*, and claims 3 and 4 are now more particularly directed toward the treatment of a cancer “selected from colon, breast, prostate, lung and skin.”

It is respectfully submitted that the specification as filed enables the skilled person to practice this invention as now claimed. As the specification notes at page 3, lines 9 *et seq.*, AZD2171 is a known compound and a very potent inhibitor of KDR and Flt-1 tyrosine kinase activity and has been shown to elicit broad-spectrum anti-tumour activity in a range of models following once-daily oral administration. It is further noted at page 4, line 11 *et seq.*, ZD1839, also known as Iressa™, is a known epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) and is an inhibitor of the proliferation of cancer tissue (page 6, lines 11-14).

A normal dosage regimen for AZD2171 is disclosed at, e.g., page 15, lines 24-29, and a dosage regimen for ZD1839 is noted at page 15, lines 30-33. Known practices and dosages of ionising radiation for radiotherapy are disclosed at page 16, lines 1-12. As further noted at page 16, lines 13-19:

As stated above the size of the dose of each therapy which is required for the therapeutic or prophylactic treatment of a particular disease state will necessarily be varied depending on the host treated, the route of administration and the severity of the illness being treated. Accordingly the optimum dosage may be determined by the practitioner who is treating any particular patient. For example, it may be necessary or desirable to reduce

the above-mentioned doses of the components of the combination treatments in order to reduce toxicity.

Thus, it is common practice in cancer therapy that the dose for a given patient must be titrated to find the appropriate dose for that patient and the cancer being treated.

Further guidance and enabling support for the administration of combinations as presently claimed is found in the tests described in the specification at pages 17-18 (Human A431 vulval carcinoma tumour xenografts in *Nude* mice) and at pages 18-19 (MMTV-neu transgenic model), in which the animals form multiple tumours in each mammary gland. It is respectfully that with the above guidance and the skill and knowledge already possessed by the skilled person in this art, such skilled person is enabled to practice the invention as presently claimed without undue experimentation.

Moreover, enablement of the claimed invention is further *confirmed* by a number of other studies detailed in the literature references that are attached hereto, which show the combination of the invention is surprisingly beneficial in a number of other tumour types, as follows:

In **lung cancer**, the Examiner's attention is drawn to O'Reilly MS, Furutani K, Wu W, Onn A, Ryan A, Jürgensmeier JM, Komaki R, Herbst R, Targeted therapy against VEGFR and/or EGFR signaling with AZD2171, vandetanib, and gefitinib as part of a combined modality approach for the treatment of non-small-cell lung cancer. *J Thorac Oncol* 2007;2 (4S):abstract A5-02. The Examiner's attention is particularly drawn to lines 22-23 in the abstract and slide 10 in the presentation.

The Examiner's attention is also drawn to Wu W, Fujitaka K, Mandal J, Imagumbai T, Ryan A, Jurgensmeier J, Fidler IJ, O'Reilly MS and Herbst RS, AZD2171, an oral, highly potent VEGFR signaling inhibitor, in combination with gefitinib or paclitaxel: results of a study in an orthotopic human lung adenocarcinoma model. *Clin Cancer Res* 2005;11: abstract B7, and particularly to the table of data on the second page under "Results," demonstrating the relative effectiveness in this model of the therapeutic agents individually and in combination.

Head and Neck Cancer

The Examiner's attention is drawn to Bozec, A., Formento, P., Lassalle, S., Lippens, C., Hofman, P. and Milano, G. (2007) *Br. J. Cancer* 97. 65-72, Dual inhibition of EGFR ad VEGFR

pathways in combination with irradiation: antitumour supra-additive effects on human head and neck cancer xenografts, particularly to Figure 1 in the publication, particularly the data that was observed with the three way combination including radiotherapy

In summary of the above, although Applicant disagrees with the Examiner's assertion that this application is not enabled for the treatment of cancer *per se*, the claims have been amended above to recite the specific cancer types of colon, breast, prostate, lung and skin. It is therefore respectfully submitted that the presently amended claims are clearly enabled in view of the above discussion of the specification disclosure and data, and the confirmation provided by the disclosure and data provided in the above-noted literature references. Withdrawal of this enablement rejection is therefore respectfully requested.

Claim Rejections - 35 USC § 112, 1st Paragraph

Claims 1-2 and 9-10 are rejected under 35 U.S.C. 112, first paragraph, as allegedly failing to comply with the written description requirement. Specifically, the Examiner asserts that the claims are directed to "the production of an antiangiogenic and/or vascular permeability reducing effect," but fail to disclose what the reducing effect is. Although Applicant respectfully disagrees with the Examiner's assertion, in an effort to advance prosecution of this application to allowance, claims 1-2 and 9-10 have been cancelled, thereby obviating this ground for rejection.

Claim Rejections - 35 USC § 112, 2nd Paragraph

The rejection of claims 9-14 as being in a "use" format not generally accepted under US practice has been obviated by the cancellation of these claims.¹

The rejection of claims 1-6 as being indefinite by reason of the recitation of the phrase "such as" has been obviated by the cancellation of claims 1, 2, 5 and 6, and overcome with respect to claims 3 and 4 by the deletion of the "such as" phrase from these claims by the above amendments.

Withdrawal of these section 112, 2nd paragraph grounds for rejection is therefore respectfully requested.

¹ The Examiner recites in this rejection that these claims "provide for the use of AZD2171 and ZD6126 ..." However, the Examiner is reminded that the present application relates to methods and combinations involving AZD2171 and ZD1839 (otherwise known as Iressa), not ZD6126 as stated by the Examiner here and elsewhere in the Action. This error is not material to this rejection inasmuch as these claims have been cancelled.

Claim Rejections - 35 USC § 101

The rejection of claims 9-14 under 35 U.S.C. § 101 as being in a “use” format not generally accepted under US practice has been obviated by the cancellation of these claims.

Claim Rejections - 35 USC § 103

At pages 9-11, claims 1-8 are rejected under 35 USC 103(a) as being unpatentable over US 2003/0055024 ('024) in view of WO 01/74360 ('360) and US 6,420,335 ('335). This ground for rejection is respectfully traversed for the reasons explained below.

In explaining how these documents support this obviousness rejection, the Examiner notes that the '024 reference “discloses the use of a vascular-damaging agent (i.e., an antiangiogenic, in particular ZD6126) in the manufacture of a medicament for administration in divided doses, optionally with a pharmaceutically acceptable excipient or carrier (§ 0017-20), for the use in the production of a vascular-damaging effect in a human (abstract) particularly a method for the treatment of a cancer involving a solid tumor (§ 0001)” (emphasis added). The Examiner acknowledges that the '024 reference does not “expressly disclose” administering together with AZD2171 and/or ionizing radiation. Therefore in support of the asserted *prima facie* obviousness the '360 reference is relied upon for the disclosure of AZD2171 as a preferred antiangiogenic, and relies upon the '335 reference as disclosing a “combination therapy using ionizing radiation and antiangiogenic factors.” (Action at pages 9-10). However, throughout this entire rejection there is no mention of ZD1839 or its combination with AZD2171, optionally in conjunction with ionising radiation, which is the combination of the present claims.

Similarly, at pages 11-14, claims 1-8 are rejected under 35 USC 103(a) as being unpatentable over US 2003/0055024 ('024) in view of US W000/47212 ('212) and US 6,420,335 ('335). This ground for rejection is also respectfully traversed for the reasons explained below.

Throughout this rejection as well, the '024 reference is relied upon for its disclosure of ZD6126 (which is *not* a part of the combination of the present claims), and there is no mention of ZD1839 (which *is* a required component of the presently claimed invention).

Inasmuch as it is apparent that the Examiner mistakenly formulated this obviousness rejection, and selected the applied references based on the mistaken belief that the present invention was directed toward a combination of AZD2171 and ZD6126, and makes no mention of any combination of AZD2171 with ZD1839 as presently claimed, Applicant is unable to

meaningfully address this rejection except to point out that *prima facie* obviousness has not been established inasmuch as the rejection omits a key component of the claimed combination.

At pages 14-16 of the Action, claims 1-8 are rejected under 35 U.S.C. 103(a) as being unpatentable over Hennequin et al (WO 01/32651) in view Magne et al. (British Journal of Cancer, vol 86, No 5, 4 March 2002, pp 819-827). This ground for rejection is also respectfully traversed for the reasons explained below. In explaining this rejection the Examiner characterizes claims 1-6 as being “drawn to a method for the treatment of cancer and a method for the production of an antiangiogenic and/or vascular permeability reducing effect in a warm-blooded animal, which comprises administering ZD6474 and ZD1839, optionally with an effective amount of ionizing radiation” and characterizes claims 7 and 8 as being “drawn to a pharmaceutical composition and kit comprising ZD6474 and ZD1839” (emphasis added). However, the present claims are directed toward a combination of AZD2171 and ZD1839, optionally in conjunction with ionising radiation.

Inasmuch as it is apparent that the Examiner mistakenly formulated this obviousness rejection, and selected the applied references based on the mistaken belief that the present invention was directed toward a combination of ZD6474 and ZD1839, and makes no mention of any combination of AZD2171 with ZD1839 as presently claimed, Applicant is unable to meaningfully address this rejection except to again point out that *prima facie* obviousness has not been established inasmuch as the rejection omits a key component of the claimed combination.

For the foregoing reasons, it is submitted that the Examiner has failed to make out a case of *prima facie* obviousness, and therefore it is respectfully requested that all grounds for rejection under 35 U.S.C. § 103 be withdrawn.

Claim Rejections - Obviousness-Type Double Patenting

Claims 1-14 are provisionally rejected under 35 U.S.C. § 101 as claiming the same invention as that of claims 1-14 of copending Applications No. 10/563,440 and 10/523,838. The Examiner correctly points out that this is a *provisional* double patenting rejection since the claims asserted to be conflicting have not in fact been patented. Nevertheless, it is respectfully submitted that neither the claims nor even the disclosed inventions of these two applications are

“conflicting” with the presently claimed invention, and that therefore this obviousness-type double patenting rejection should be withdrawn.

Specifically, application 10/563440 is pending before Examiner Chris Simmons in Group 1612, and is currently awaiting examiner action on applicant’s response to a non-final action. However, application 10/563440 relates to a combination of AZD2171 and ZD6126, whereas the presently claimed invention relates to a combination of AZD2171 and ZD1839, optionally in conjunction with ionising radiation. Clearly there is no overlap or “conflict” between the presently claimed invention and the invention disclosed and claimed in application 10/563440. It is therefore respectfully requested that this obviousness-type double patenting rejection be withdrawn.

Application 10/523,838 is pending before Examiner Christopher Stone in Group 1614, and is currently awaiting examiner action on applicant’s response to a non-final action. However, application 10/523,838 relates to the combination of ZD6474 and ZD1839, whereas the presently claimed invention relates to a combination of AZD2171 and ZD1839, optionally in conjunction with ionising radiation. Clearly there is no overlap or “conflict” between the presently claimed invention and the invention disclosed and claimed in application 10/523,838. It is therefore respectfully requested that this obviousness-type double patenting rejection be withdrawn.

Technically Related Pending Applications of Applicant’s Assignee

The Examiner’s attention is drawn to the following co-pending U.S. non-provisional applications of Applicant’s assignees which disclose and claim combination therapy including *either* AZD2171 *or* ZD1839.

The following table lists such applications including AZD2171:

US Appln	Date US Filed	US Pub. #	PCT Pub. #	Combination with	Current Status
10/240413	01 Oct 2002	20030144298 31 Jul 2003	WO2001/74360 10 Oct 2001	Anti-hypertensive	Assigned to Examiner Charlesworth E Rae in GAU 1611; non-final Action mailed 01-02-2008.
10/563440	05 Jan 2006	20060160775 20 Jul 2006	WO 2005/004871 20 Jan 2005	ZD6126	Assigned to Examiner Chris E Simmons in GAU 1612; Response to Non-Final Office Action Entered and Forwarded to Examiner, 05-02-2008
10/594235	25 Sep 2006	20080113039 29 May 2008	WO 2005/092384 06 Oct 2005	Platinum anti-tumor agent, optionally IR	Assigned to Examiner Ardin H Marschel in GAU 1614; Docketed - Ready for Examination
10/594233	25 Sep 2006	20080125447 29 May 2008	WO 2005/092303 06 Oct 2005	CPT-11 and/or 5-FU	Assigned to Examiner Ardin H Marschel in GAU 1614; Docketed - Ready for Examination
10/594234	25 Sep 2006	20070135462 14 June 2007	WO 2005/092385 06 Oct 2005	Taxane, optionally IR	Assigned to Examiner Charlesworth E Rae in GAU 1611; Response to Non-Final Office Action Entered and Forwarded to Examiner, 04-17-2008
11/663912	27 Mar 2007	20080015205 17 Jan 2008	WO 2006/035203 06 Apr 2006	Imatinib [Gleevec]	Assigned to Examiner James D. Anderson in GAU 1614; Docketed - Ready for Examination.
11/994824	04 Jan 2008		WO 2007/003933 11 Jan 2007	Gemcitabane [Gemzar]	Application Undergoing Preexam Processing; Not yet assigned or published

The following table lists such applications including ZD1839:

US Appln	Date US Filed	US Pub. #	PCT Pub. #	Combination	Current Status
10/511744	18 Oct 2004	20050215530 29 Sep 2005	WO 03/088971 30 Oct 2003	ZD6126	Assigned to Examiner Alicia R Hughes in GAU 1614; Final Rejection Mailed 01-04-2008
10/523838	08 Feb 2005	20050245549 03 Nov 2005	WO 2004/014426 19 Feb 2004	ZD6474	Assigned to Examiner Christopher R Stone in GAU 1614; Response to Non-Final Office Action Entered and Forwarded to Examiner 05-03-2008
10/530794	08 Apr 2005	20060122180 08 Jun 2006	WO2004/035057 29 Apr 2004	AZD4054	Assigned to Examiner Marcos L Sznajdman, Response to Non Final Action Mailed 16 May 2008

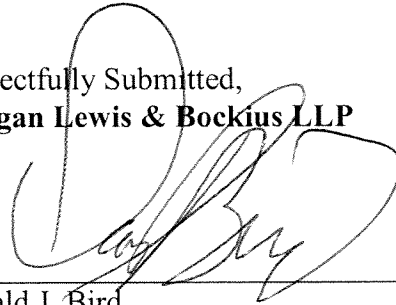
US Appln	Date US Filed	US Pub. #	PCT Pub. #	Combination	Current Status
11/597940	29 Nov 2006	20070254893 01 Nov 2007	WO2005/117888 15 Dec 2005	AZD0530	Assigned to Examiner Yong Soo Chong in GAU 1617; docketed, ready for action.

Conclusion

All grounds for rejection having been addressed above and either obviated or overcome by the above amendments and/or remarks, it is believed that all claims are now in condition for allowance, and a Notice to that effect is respectfully requested.

EXCEPT for issue fees payable under 37 C.F.R. § 1.18, the Director is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 C.F.R. §§ 1.16 and 1.17 which may be required, including any required extension of time fees, or credit any overpayment to Deposit Account 50-0310. This paragraph is intended to be a **CONSTRUCTIVE PETITION FOR EXTENSION OF TIME** in accordance with 37 C.F.R. § 1.136(a)(3).

Respectfully Submitted,
Morgan Lewis & Bockius LLP



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Targeted therapy against VEGFR and/or EGFR signaling with AZD2171, vandetanib, and gefitinib as part of a combined modality approach for the treatment of non-small-cell lung cancer

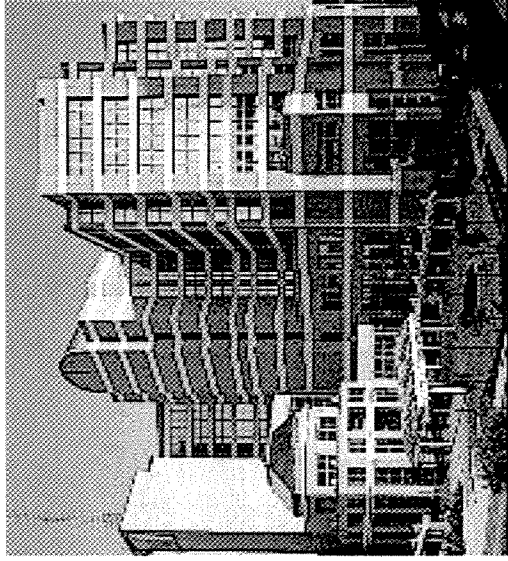
O'Reilly, Michael S.¹ Furutani, Kazuhisa¹ Wu, Wenjuan¹ Onn, Amir¹ Ryan, Anderson² Jürgensmeier, Juliane M.² Komaki, Ritsuko¹ Herbst, Roy S.¹
¹ MD Anderson Cancer Center, Houston, TX, USA ² AstraZeneca, Cheshire, UK

Background: The current challenge in NSCLC is to optimize available therapeutic strategies by incorporating new agents into existing treatment regimens. Strategies that target key signaling pathways offer great potential and two validated therapeutic targets are VEGF and EGF and their receptors. To study the potential therapeutic efficacy of lung cancer treatments we have developed orthotopic lung adenocarcinoma models that mimic clinical patterns of NSCLC growth, which are sensitive (H441) or highly resistant (PC14) to EGFR inhibition. EGFR is expressed in both models, though EGFR ligand expression (TGF- α /EGF) was observed only for the H441 lung adenocarcinomas and was associated with resultant endothelial expression and activation of EGFR.

Methods: Human lung adenocarcinoma cells (PC14 or H441) were injected into the left lungs of nude mice with lung tumors evident within 14 days. Tumor-bearing mice (10/group) were treated with (A) AZD2171 (RECENTIN™), a highly potent and selective inhibitor of VEGFR-1, -2, and -3 (6 mg/kg/day orally); (B) vandetanib (ZACTIMA™), a selective inhibitor of VEGFR-2 and EGFR (25 mg/kg/day orally); (C) AZD2171 plus paclitaxel (200 μ g/week ip); (D) vandetanib plus paclitaxel; (E) gefitinib (IRESSA™), a highly selective inhibitor of EGFR (25 or 75 mg/kg/day orally); (F) gefitinib plus AZD2171; or (G) appropriate drug vehicles as controls. All animals were then sacrificed and assessed for tumor burden, pleural effusion and lymphatic metastasis. Tumor tissues were then subjected to immunohistochemical analyses.

Results: Vandetanib or AZD2171 treatment substantially reduced lung tumor burden in both the H441 and PC14 tumor models, and prevented mediastinal adenopathy and pleural effusion relative to controls. In both models, the antitumor and antimetastatic effects of AZD2171 and vandetanib were markedly enhanced when they were combined with paclitaxel. Immunohistochemical analyses of H441 lung adenocarcinoma revealed that tumor and endothelial cell VEGFR activation was inhibited by vandetanib or AZD2171 therapy alone and in combination with paclitaxel in lung tumors. Vandetanib or AZD2171 inhibited tumor angiogenesis and enhanced the antivascular and antitumor effects of paclitaxel. H441 tumors, but not PC14 tumors, were sensitive to EGFR signaling inhibition by gefitinib, which enhanced the antitumor and antivascular effects of AZD2171 in a dose-dependent fashion for H441 lung adenocarcinomas. Immunohistochemical evaluation of H441 lung adenocarcinomas showed that angiogenesis, tumor cell proliferation, and expression of proangiogenic and invasive molecules were substantially reduced by treatment with AZD2171 in combination with gefitinib compared with either agent alone.

Conclusions: These studies demonstrate that VEGFR signaling inhibition by AZD2171 or vandetanib inhibits tumor growth and angiogenesis in orthotopic human lung adenocarcinoma models. The antitumor and antivascular effects of AZD2171 or vandetanib were substantially enhanced when they were combined with paclitaxel. EGFR signaling inhibition enhanced the therapeutic efficacy of VEGFR signaling inhibition for H441 lung tumors, which express both EGFR and its ligand(s). These findings provide mechanistic insights into the biology underlying the beneficial combination of AZD2171 or vandetanib with paclitaxel in lung cancer, and suggest that a customized approach for combined EGFR and VEGFR signaling inhibition based upon EGFR expression and activation in lung tumors and endothelial cells warrants further investigation.



Targeted therapy against VEGFR and/or EGFR signaling with cediranib (RECENTIN™, AZD2171), vandetanib (ZACTIMA™, ZD6474), and gefitinib (IRESSA™, ZD1839) as part of a combined modality approach for the treatment of non-small-cell lung cancer

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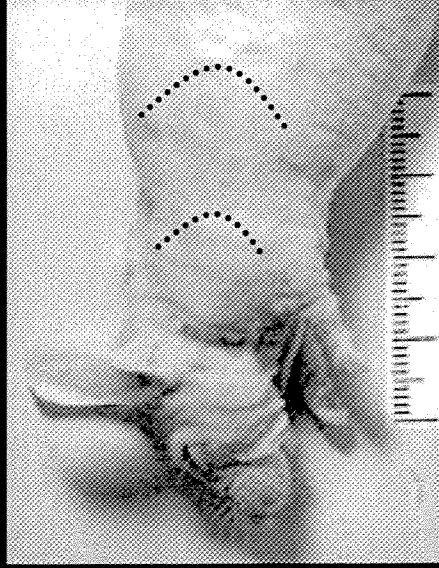
THE UNIVERSITY OF TEXAS
MD ANDERSON
CANCER CENTER
Making Cancer History™

Development of an orthotopic lung cancer model: human lung adenocarcinoma

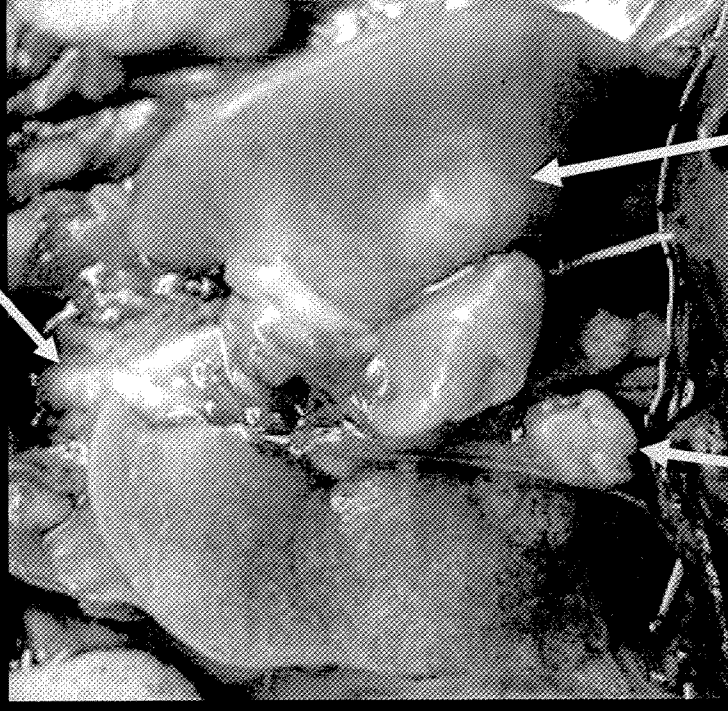
Anesthesia: sodium pentobarbital

Site of injection: left lung

Total cell number: 5×10^5
(37.5 μ l HBSS with 37.5 μ l Matrigel)

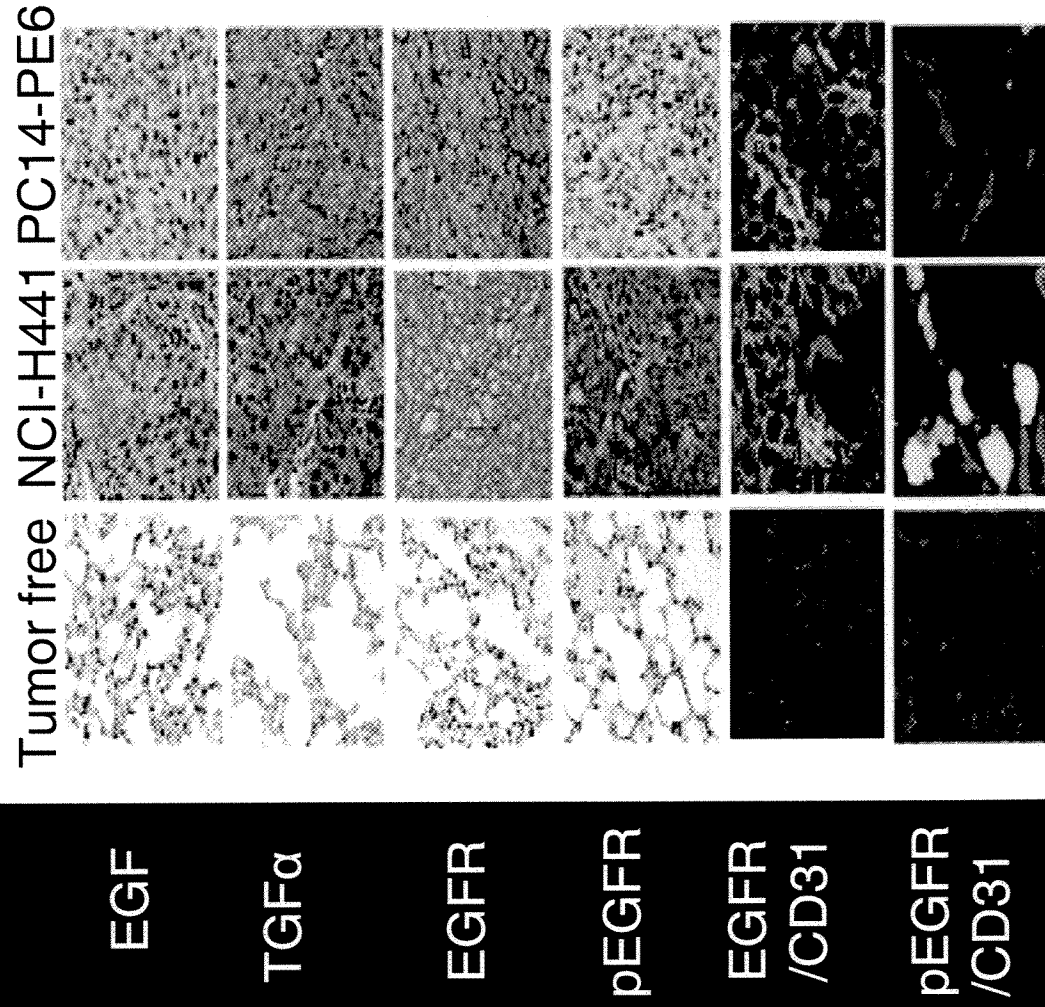


Trachea



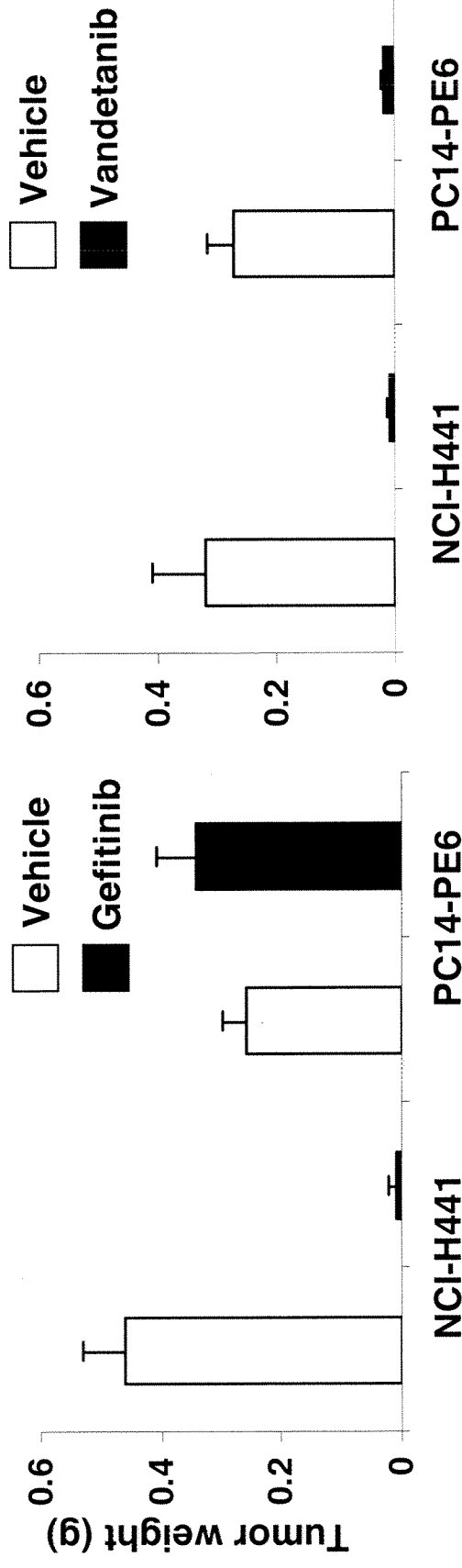
Primary tumor
Lymphatic metastasis

Tumor cell and endothelial cell expression and activation of EGFR and its ligands in orthotopic NCI-H441 or PC14-PE6 human lung adenocarcinomas



Treatment of orthotopic PC14-PE6 or NCI-H441 human lung adenocarcinomas with gefitinib or vandetanib

NCI-H441 cells are EGFR positive and EGF/TGF α positive
PC14-PE6 cells are EGFR positive and EGF/TGF α negative



Gefitinib (50 mg/kg) or vandetanib (25 mg/kg) were administered by oral gavage once daily starting 7 days after lung tumor injection

Treatment of orthotopic PC14-PE6 (rapidly progressive model) or NCI-H441 (slowly progressive model) human lung adenocarcinomas with cediranib and/or paclitaxel

Paclitaxel/
cediranib

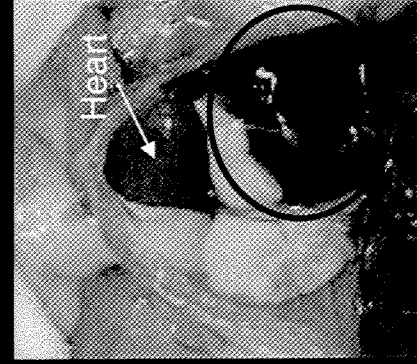
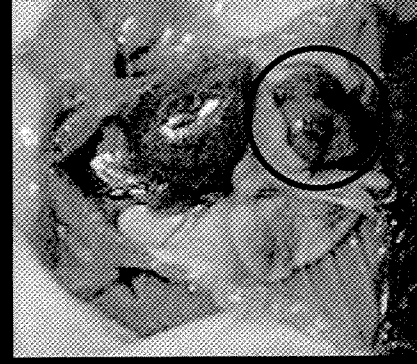
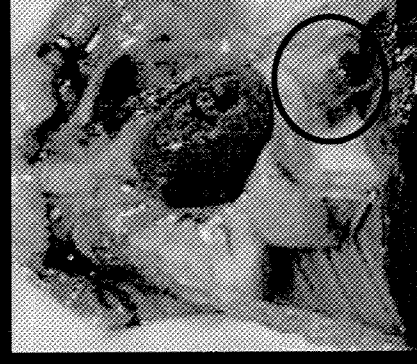
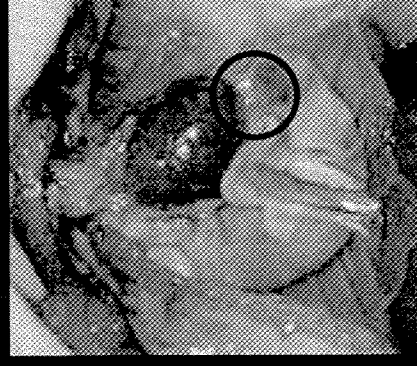
Cediranib

6 mg/kg/day po

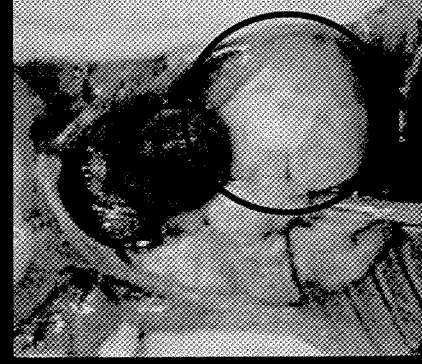
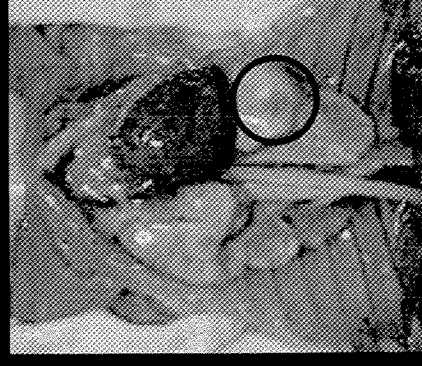
Paclitaxel

200 µg weekly ip

Vehicle



PC14-PE6



NCI- H441

Treatment of orthotopic NCI-H441 human lung adenocarcinomas with cediranib and/or paclitaxel

Treatment group	Tumor incidence	Lung weight (mg) Median (range)	Tumor volume (mm ³) Median (range)	Lymphatic metastasis	Distant metastasis
Vehicle	10/10	1046 (295–1760)	746 (97–1337)	6/10	2/10
Paclitaxel	10/10	389 (220–1259)	194 (20–837)	7/10	2/10
Cediranib	10/10	242 (199–591)	37 (4–224)	4/10	0/10
Cediranib/ paclitaxel	10/10	237 (180–363)	12 (2–151)	2/10	0/10

Paclitaxel (200 µg weekly ip) and/or cediranib (6 mg/kg/day po) treatment was started 2 weeks after lung tumor implantation
Treatment continued for 107 days until control mice became moribund

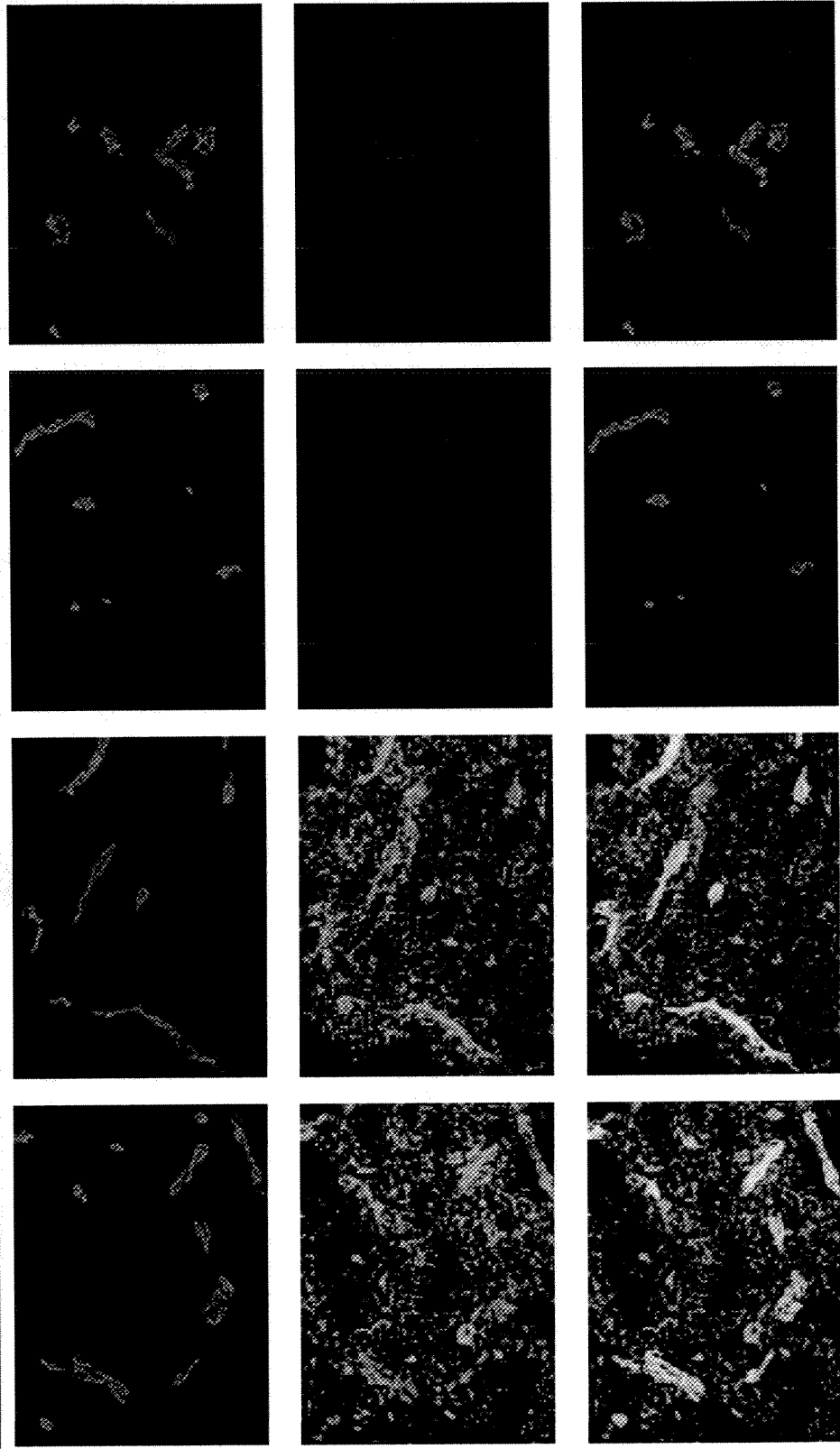
Treatment of orthotopic NCI-H441 human lung adenocarcinomas with cediranib and/or paclitaxel: VEGFR-2 activation for tumor cells and tumor-associated endothelial cells

Cediranib/
paclitaxel

Cediranib
6 mg/kg/day po

Paclitaxel
200 µg weekly ip

Vehicle



CD31

pVEGFR2

CD31/
pVEGFR2


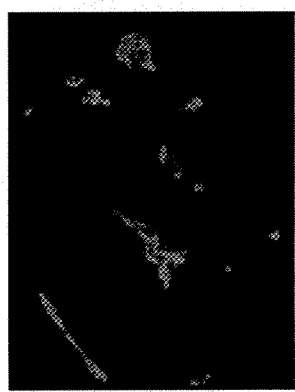


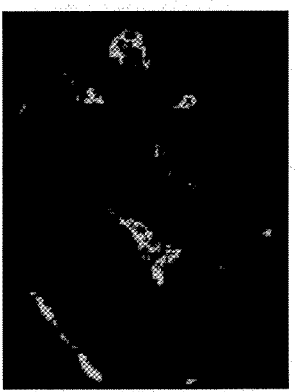
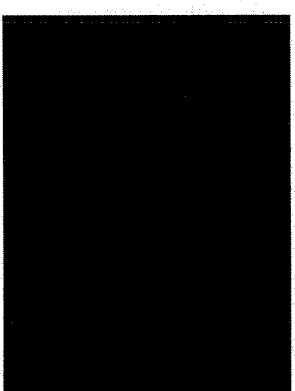
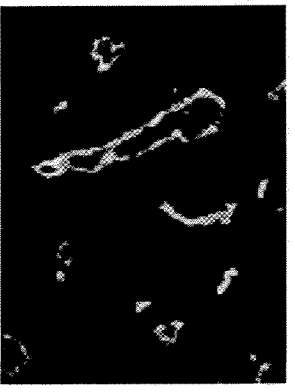


Treatment of orthotopic PC14-PE6 human lung adenocarcinomas with cediranib and/or paclitaxel

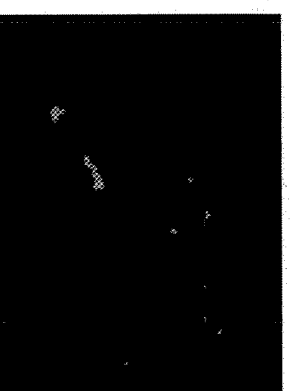
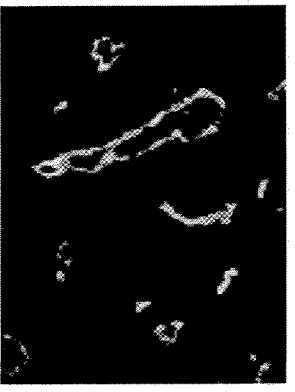
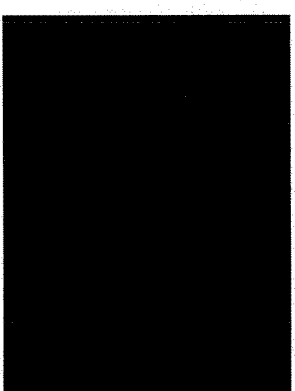
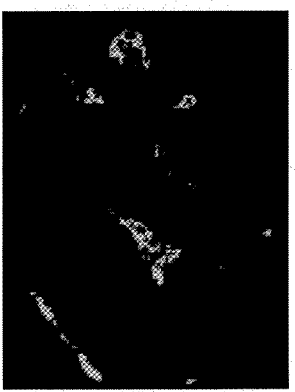
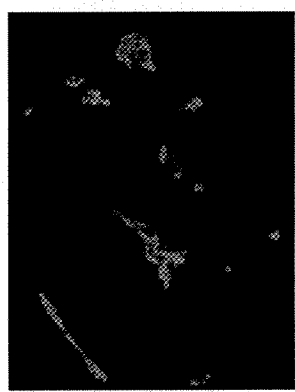
Treatment group	Tumor incidence	Lung weight (mg) Median (range)	Tumor volume (mm ³) Median (range)	Lymphatic metastasis	Pleural effusion (μl) Median (range)
Vehicle	10/10	1176 (488–1659)	1148 (539–1946)	6/10	550 (450–1000)
Paclitaxel	10/10	808 (284–1392)	516 (18–1593)	4/10	30 (0–950)
Cediranib	10/10	331 (283–475)	148 (73–354)	1/10	0 (0–0.45)
Cediranib/ paclitaxel	10/10	230 (193–248)*	35 (4–127)*	0/10	0 (0–0.02)

Paclitaxel (200 μg weekly ip) and/or cediranib (6 mg/kg/day po) treatment was started 2 weeks after lung tumor implantation

* $P < 0.01$ as compared with control, paclitaxel and cediranib groups

Treatment of orthotopic PC14-PE6 human lung adenocarcinomas with cediranib and/or paclitaxel: VEGFR-2 activation for tumor cells and tumor-associated endothelial cells

	Paclitaxel 200 µg weekly ip	Cediranib 6 mg/kg/day po	Cediranib/ paclitaxel
Vehicle			
CD31			
pVEGFR2			
CD31/ pVEGFR2			

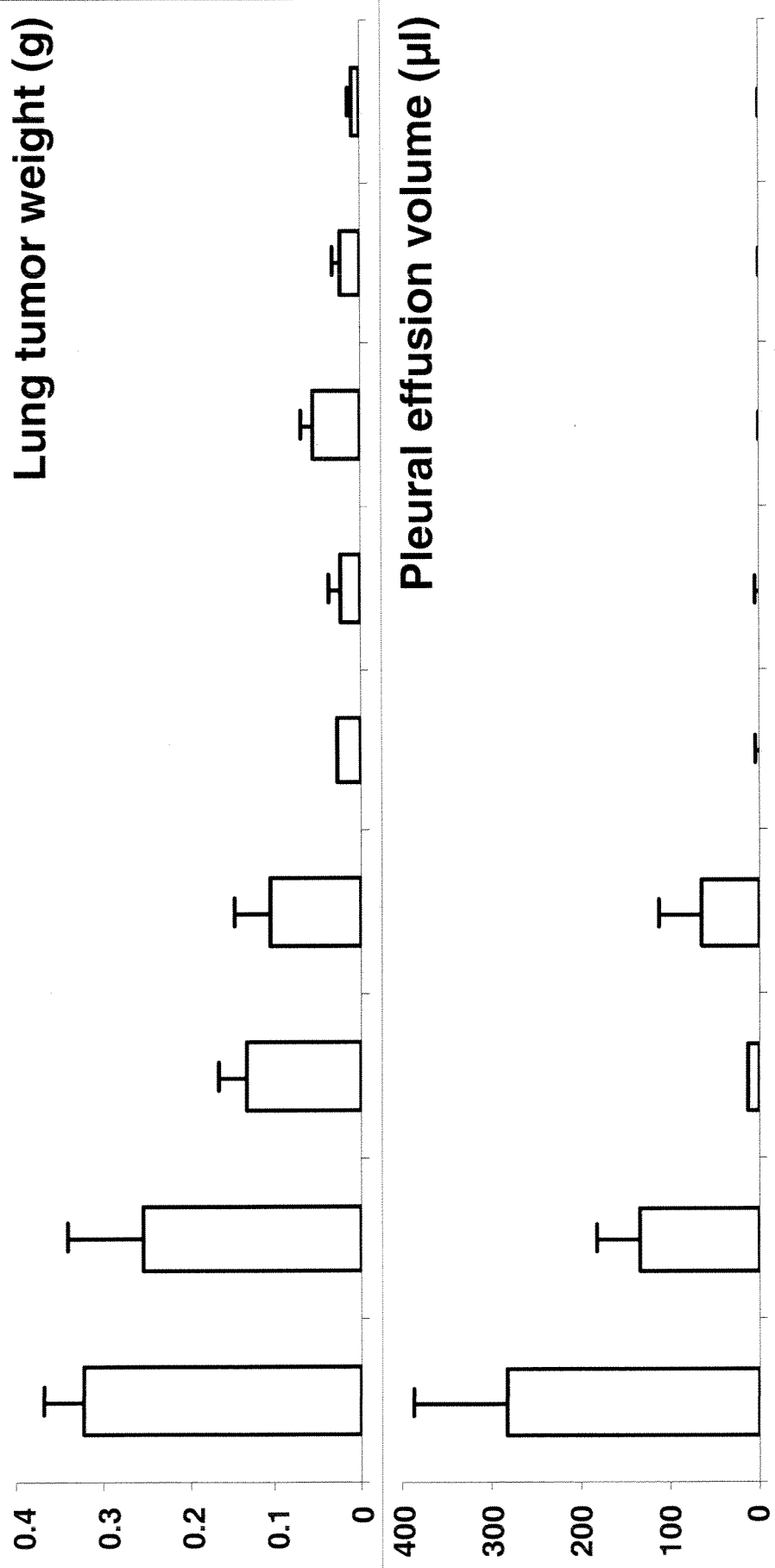


CD31

pVEGFR2

CD31/
pVEGFR2

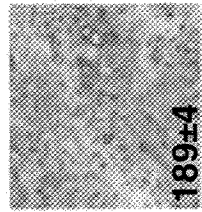

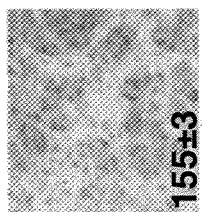
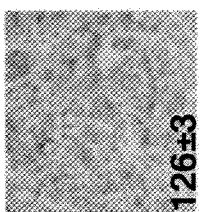
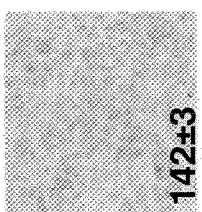
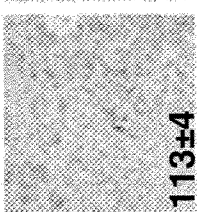
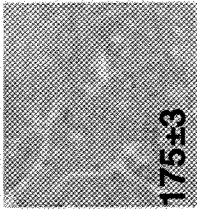
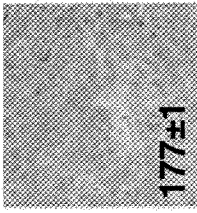
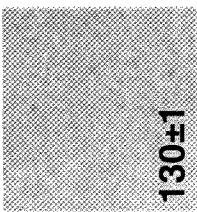
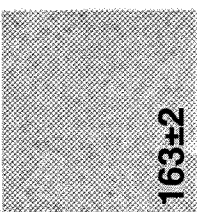
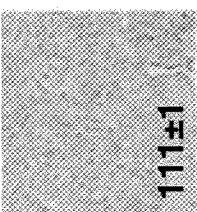
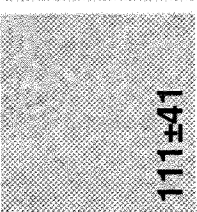
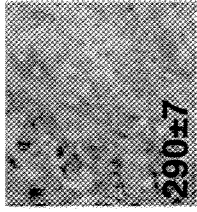
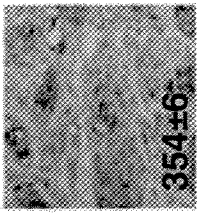
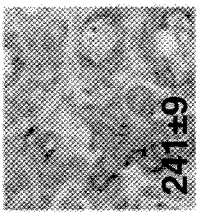
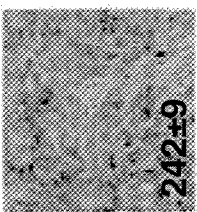
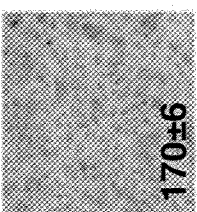
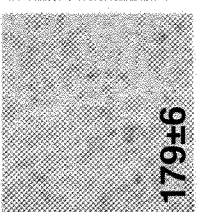
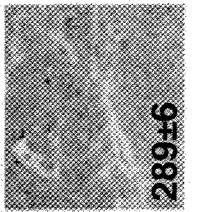
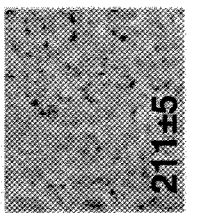
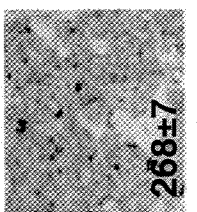
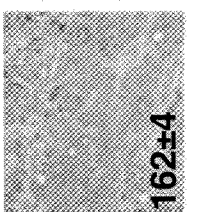
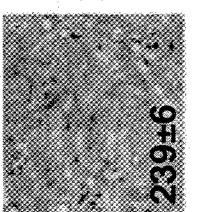
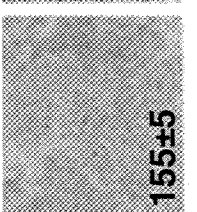

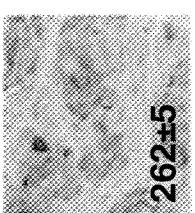
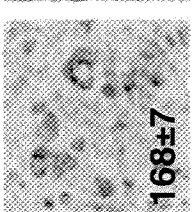
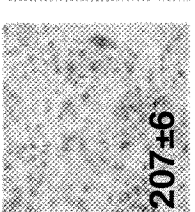
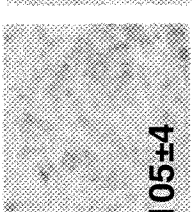
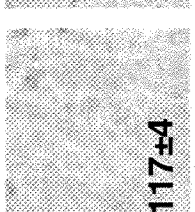
Treatment of orthotopic NCI-H441 human lung adenocarcinomas with paclitaxel, cediranib, gefitinib, and/or vandetanib



Vehicle	Paclitaxel 150 µg ip weekly	Cediranib 6 mg/kg po daily	Gefitinib 25 mg/kg po daily	Gefitinib 75 mg/kg po daily	Vandetanib 25 mg/kg po daily	Cediranib + paclitaxel	Cediranib + gefitinib 25 mg/kg	Cediranib + gefitinib 75 mg/kg
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Treatment of orthotopic NCI-H441 lung adenocarcinomas with paclitaxel, cediranib, gefitinib, and vandetanib: immunohistochemistry

Paclitaxel 150 µg/week ip **Cediranib** 6 mg/kg/day po **Gefitinib** 25 mg/kg/day po **Vandetanib** 25 mg/kg/day po

	Vehicle	Paclitaxel	Cediranib	Gefitinib	Cediranib/ paclitaxel	Gefitinib/ vandetanib
VEGF	 189±4	 184±3	 155±3	 126±3	 142±3	 113±4
pVEGFR-2	 175±3	 177±1	 130±1	 163±2	 111±1	 111±4
EGF	 290±7	 354±6	 241±9	 242±9	 170±6	 179±6
pEGFR	 289±6	 211±5	 268±7	 162±4	 239±6	 155±5
bFGF	 216±5	 262±5	 168±7	 207±6	 105±4	 117±4

Immunohistochemical reaction intensity is expressed as mean ± standard deviation

Conclusions

- Both ligand and receptor expression by tumor cells and tumor associated endothelial cells should be considered when designing therapeutic strategies directed against VEGFR and/or EGFR
- Targeted therapy directed against all three VEGFRs is broadly effective against orthotopic lung adenocarcinoma and enhances the anti-tumor activity of paclitaxel
- The combined targeting of VEGFR and EGFR should be considered for lung cancers that respond to EGFR inhibition
- The translation of biologically targeted therapies into the clinic is now inevitable and within our grasp

AZD2171, an oral, highly potent VEGFR signaling inhibitor, in combination with gefitinib or paclitaxel: results of a study in an orthotopic human lung adenocarcinoma model

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Introduction

- Lung cancer is a leading cause of cancer death worldwide and the current treatment options available to patients with advanced disease remain limited.
- Angiogenesis plays an essential role in tumor growth and metastasis, and inhibition of angiogenesis provides a novel approach for treating human lung cancer and other cancers.^{1,2}
- Vascular endothelial growth factor-A (VEGF) is an important growth factor for angiogenesis that regulates endothelial cell proliferation, migration and vascular permeability by binding to the VEGF receptors VEGFR-1 (Flt-1) and VEGFR-2 (KDR). VEGFR-3 (Flt-4) has a critical role in lymphangiogenesis.⁴
- AZD2171 is a highly potent, orally active inhibitor of VEGF signaling. Preclinical investigations have demonstrated that AZD2171 inhibited VEGFR-2 tyrosine kinase at subnanomolar concentrations (IC_{50} < 1 nmol/L) and also potentially inhibited VEGFR-1 (IC_{50} = 5 nmol/L) and VEGFR-3 (IC_{50} < 3 nmol/L) tyrosine kinase activity.⁵
- AZD2171 has been shown to inhibit VEGF-induced human umbilical vein endothelial cell proliferation *in vitro* and inhibit VEGF signaling, angiogenesis and xenograft tumor growth *in vivo*.³
- The purpose of this study was to examine the antiangiogenic, antitumor, antineoplastic and antitumor effects of AZD2171 in combination with the EGFR inhibitor gefitinib (IRESSA[®]) or cytotoxic chemotherapy (paclitaxel) in a clinically relevant, murine, orthotopic model of human lung cancer.

Methods

Cell lines and reagents

- NCI-H441 cells were obtained from the ATCC. AZD2171 and gefitinib were provided by AstraZeneca (Macclesfield, UK). Paclitaxel was obtained from Bristol-Myers Squibb (Princeton, NJ).

In vivo orthotopic lung cancer model

- A suspension of 5×10^5 lung adenocarcinoma (NCI-H441) cells mixed with 20% Matrigel was injected into the left lung of nude mice. This model develops primary tumors within 5 days, which metastasize to the lymph nodes and chest wall and produce pleural effusion.⁶

Treatment regimens

- Treatment with AZD2171 alone (6 mg/kg/day p.o.), gefitinib alone (25 mg/kg/day p.o.), paclitaxel alone (150 µg/mouse/week, i.p.) and AZD2171 in combination with gefitinib or paclitaxel was initiated 5 days after tumor injection (n=10 mice/group).

- Mice were euthanized when the control group began to show signs of the onset of morbidity. The lung tumor weight, pleural effusion and pleural invasion were measured, and tumor tissue removed.

Immunohistochemistry

- Excised tumors and adjacent tissues were frozen or formalin fixed. CD31 expression was determined in frozen tissue sections using rat anti-mouse CD31 MAb (Pharmingen, San Diego, CA).

- Expression of PCNA (DAKO, Copenhagen, Denmark); bFGF (IL-8, MMP-2, MMP-9, EGF, TGF- α , VEGF (Santa Cruz Biotechnology, Santa Cruz, CA); EGFR (Zymed, South San Francisco, CA); activated-EGFR (Biosource International, Camarillo, CA); VEGFR-2 (Santa Cruz Biotechnology), and activated VEGFR-2 (Oncogene, Cambridge, MA) were determined in formalin-fixed, paraffin-embedded tissue sections.

Quantification of MVD, PCNA and IHC reaction density

- For quantification of microvessel density (MVD) and PCNA-positive reaction in tumors, 10 random 0.159 mm² fields at $\times 100$ magnification were captured for each tumor after staining and quantified using Scion software based on the NIH Image program for Macintosh (Scion Corp, Frederick, MD).
- For quantification of the immunohistochemistry (IHC) reaction intensity, the absorbance of positive cells from each of the tumor tissues was measured in 10 random 0.039 mm² fields at $\times 200$ magnification using Optimas Image Analysis software.
- The samples were not counterstained so that the absorbance was attributable solely to the product of the IHC reaction.
- The receptor or cytoplasmic immunoreactivity was evaluated by computer-assisted image analysis and the expression in tumor tissue was expressed as a percentage of that observed in tumor-free tissue.

Statistical analysis

- The data were analyzed by Student's t-test (two-tailed).

Results

- The suppression of tumor growth, pleural effusion and metastasis to lymph nodes and chest wall in the orthotopic lung model was greater in mice treated with AZD2171 plus gefitinib or paclitaxel, compared with each agent as monotherapy (Table 1).
- Immunohistochemical evaluation of primary lung tumors showed that MVD, cell proliferation, and levels of proangiogenic factors (VEGF, bFGF, TGF- α and IL-8) and invasive molecules (MMP-2 and MMP-9) were substantially reduced by treatment with AZD2171 in combination with gefitinib or paclitaxel (Table 2, Figures 1 and 2).

Table 1. Inhibition of tumor growth, pleural effusion and metastasis in an orthotopic model of human lung cancer (NCI-H441) by AZD2171 in combination with gefitinib or paclitaxel (mean \pm SE)

	Control	Paclitaxel (150 µg/kg, i.p., 5 days)	AZD2171 (6 mg/kg, p.o., 5 days)	Gefitinib (25 mg/kg, p.o., 5 days)	AZD2171 + paclitaxel (150 µg/kg, i.p., 5 days)	AZD2171 + gefitinib (25 mg/kg, p.o., 5 days)
Lung tumor (g)	0.32 \pm 0.06	0.26 \pm 0.09	0.13 \pm 0.03*	0.11 \pm 0.04*	0.05 \pm 0.02*	0.02 \pm 0.01*
Pleural effusion (µl)	284 \pm 107	138 \pm 50	11 \pm 3*	65 \pm 50*	0*	0*
Number of pleural tumors	8.1 \pm 1.2	6.0 \pm 2.3	4.9 \pm 1.0	4.8 \pm 1.9	1.7 \pm 1.2*	3.1 \pm 0.8*
Incidence of lymph node metastases (%)	100	70	100	60	20	50

*p<0.01, according to Student's two-tailed t-test

†p<0.05

Table 2. IHC analysis of lung tumors treated with AZD2171 in combination with paclitaxel or gefitinib in a lung orthotopic model

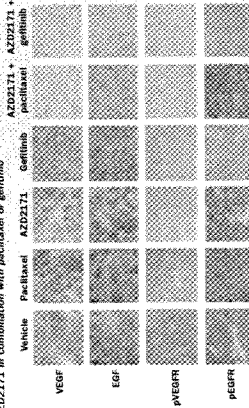
Group	pVEGFR-2	pEGFR	pVEGF	TGF- α	bFGF	IL-8	MMP-2	MMP-9	CD31	PCNA
Vehicle	179 \pm 13	269 \pm 6	183 \pm 4	290 \pm 7	271 \pm 4	219 \pm 5	351 \pm 6	340 \pm 6	31 \pm 3	92 \pm 2
AZD2171	130 \pm 11	268 \pm 7	135 \pm 3	241 \pm 9	198 \pm 5*	168 \pm 7*	238 \pm 9*	289 \pm 5	18 \pm 1*	89 \pm 4
Gefitinib	153 \pm 12	182 \pm 4	126 \pm 3	242 \pm 9	172 \pm 9*	207 \pm 6	136 \pm 2*	173 \pm 3*	17 \pm 2*	98 \pm 5*
Paclitaxel	151 \pm 11	211 \pm 10	164 \pm 3	254 \pm 6	233 \pm 9	283 \pm 5	302 \pm 7	279 \pm 7	26 \pm 1*	87 \pm 2
AZD2171 + gefitinib	111 \pm 11*	155 \pm 5*	133 \pm 4*	178 \pm 6*	157 \pm 4*	117 \pm 4*	119 \pm 2*	164 \pm 3*	8 \pm 1*	32 \pm 4*
AZD2171 + paclitaxel	111 \pm 11*	139 \pm 10*	142 \pm 9*	170 \pm 9*	145 \pm 4*	109 \pm 1*	213 \pm 6*	136 \pm 3*	14 \pm 2*	32 \pm 3*

* Mean \pm SE intensity of positive cells from each tumor tissue was measured in 10 random 0.039 mm² fields at $\times 200$ magnification using Optimas Image Analysis software and the expression in tumor tissue was expressed as a percentage of that observed in tumor-free tissue.

† Mean \pm SE ICD-1 or PCNA-positive cells in tumor-free tissue was measured in 10 random 0.159 mm² fields at $\times 100$ magnification for each tumor using Scion software based on the NIH Image program for Macintosh.

‡ p<0.05, compared with vehicle group, according to Student's two-tailed t-test.

Figure 1. Changes in VEGF, EGFR and receptor phosphorylation following treatment with AZD2171 in combination with paclitaxel or gefitinib



Conclusions

- In this orthotopic human lung adenocarcinoma model, AZD2171 was associated with significant antitumor and antitumor effects.
- Combining AZD2171 with the EGFR inhibitor gefitinib or with paclitaxel enhanced the observed antitumor and antitumor effects.
- The significant antitumor effects seen in this *in vivo* model provide an encouraging basis for the clinical evaluation of AZD2171 in lung cancer patients.

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Dual inhibition of EGFR and VEGFR pathways in combination with irradiation: antitumour supra-additive effects on human head and neck cancer xenografts

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The aim of this study was to investigate the effects of combining antiangiogenic treatment, epidermal growth factor receptor (EGFR) targeting and irradiation (RT). We evaluated AZD2171, a highly potent, orally active, vascular endothelial growth factor (VEGF) signalling inhibitor, gefitinib, an EGFR tyrosine kinase inhibitor and RT. The antitumour efficacy of these treatments, administered alone and in combination for 2 weeks, was assessed in a VEGF-secreting human head and neck tumour cell line, CAL33 that highly expresses EGFR, established as xenografts (250 mm³) in nude mice. The median time to reach a tumour volume of 1000 mm³ was significantly increased for AZD2171 or gefitinib alone compared with the control. Greater inhibition of tumour growth was seen with the combination of AZD2171 + gefitinib compared with either drug alone, and the triple combination compared with either AZD2171 + gefitinib or RT alone. The intensity of endothelial cell staining was slightly reduced by each agent given alone, and markedly diminished by the double or triple combination. The triple combination almost completely abolished cell proliferation. The marked RT-induced enhancement in the DNA-repair enzyme ERCC1 expression was totally abolished by the triple combination. This observation could help to explain the supra-additive antitumour effect produced by this combination and could provide a basis for future innovative clinical trials.

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Keywords: AZD2171; gefitinib; radiotherapy; head and neck cancer; antiangiogenic; tyrosine kinase inhibitor

Agents that target epidermal growth factor receptor (EGFR) potentially exert antitumour effects by inhibiting tumour cell proliferation and survival, as well as reducing the secretion of proangiogenic growth factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor that stimulate tumour neoangiogenesis (Perrotte *et al*, 1999; Woodburn, 1999; Ciardiello *et al*, 2001). It has been reported that EGFR targeting in tumours may also modulate the migration and formation of tube-like structures of vascular endothelial cells (Hirata *et al*, 2002). More recently, we have shown the presence of a gefitinib-sensitive functional EGFR pathway in an immortalised microvascular endothelial cell line of human origin (Bozec *et al*, 2005). Thus, in addition to direct effects on tumour cells, EGFR-targeting drugs may also impart an indirect antitumour effect through antiangiogenic activity. An optimal antiangiogenic strategy may, therefore, be to combine an EGFR signalling inhibitor with an agent that selectively targets VEGF-dependent signalling.

The novel indole-ether quinazoline, AZD2171, is a highly potent (IC₅₀ < 1 nM) ATP-competitive inhibitor of recombinant VEGF receptor-2 (VEGFR-2) tyrosine kinase *in vitro* (Wedge *et al*, 2005). AZD2171 also shows potent activity vs VEGFR-1 (IC₅₀ = 5 nM) and VEGFR-3 (IC₅₀ ≤ 3 nM). In human umbilical vein endothelial cells, AZD2171 inhibited VEGF-stimulated proliferation and VEGFR-2 phosphorylation with IC₅₀ values of 0.4 and 0.5 nM, respectively. In a fibroblast/endothelial cell coculture model of vessel sprouting, AZD2171 reduced vessel area, length and branching at sub-nanomolar concentrations. The growth of established human tumour xenografts in athymic mice was dose-dependently inhibited by chronic administration of AZD2171. Combining AZD2171 and gefitinib could potentially provide inhibitory effects on both endothelial and tumour cells. Recent preclinical studies suggest that radiotherapy (RT) in combination with antiangiogenic/vasculature-targeting agents may enhance the therapeutic ratio of ionising radiation alone (Wachsberger *et al*, 2005; Cao *et al*, 2006; Williams *et al*, 2007). Thus, the combination of AZD2171 and gefitinib with RT could also be interesting to investigate.

Tumour vasculature is a key target in the treatment of solid tumours, particularly in head and neck cancer (Le and Giaccia, 2003). In the present study, the antitumour activity of AZD2171, in combination with gefitinib and RT on a number of cellular and molecular markers in human head and neck tumour xenografts (CAL33), was investigated.

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MATERIALS AND METHODS

Chemicals

AZD2171 and gefitinib were kindly provided by AstraZeneca, Macclesfield, Great Britain. Working solutions were prepared as follows: AZD2171 (375 mg l^{-1}) and gefitinib (7.5 g l^{-1}) were suspended in 0.9% NaCl, 0.01% Tween 80. For both drugs, the concentrations were adjusted so as to include the daily dose in 0.2 ml of drug suspension. Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin and glutamine were purchased from Whittaker (Verviers, Belgium). Foetal bovine serum (FBS) was obtained from Dutscher (Brumath, France).

Cell lines

CAL33, a cell line of human head and neck origin, was obtained from our institution (Centre Antoine-Lacassagne, Nice, France). This cell line exhibits high EGFR levels ($33794 \pm 624 \text{ fmol mg}^{-1}$ protein high-affinity sites determined by ligand binding assay; Dassonville *et al*, 1993) and produces VEGF (C Onesto, CNRS-UMR6543, personal communication).

The cell line was maintained as monolayer culture in DMEM supplemented with 10% FBS v/v, 2 mM glutamic acid, $50\,000 \text{ U l}^{-1}$ penicillin and $80 \mu\text{M}$ streptomycin in a humidified incubator (Sanyo, Osaka, Japan) at 37°C in an atmosphere containing 8% CO_2 . Batches of 15×10^6 cells were frozen in FBS supplemented with 5% dimethyl sulphoxide (v/v) in advance for injection into mice. Shortly before injection, cells were thawed and suspended in Ringer lactate.

Mice

Animal experiments were performed in accordance with the regulations of our institution's ethics commission and with the United Kingdom Co-ordinating Committee on Cancer Research guidelines (Workman *et al*, 1998). Six-week-old male NMRI nude mice were purchased from Janvier laboratories (Le Genet sur Isle, France) and received s.c. inoculation in the right flank of 2×10^6 cells suspended in $100 \mu\text{l}$ of Ringer lactate ($n = 10$ per treatment condition). There were five animals per cage with food and water *ad libitum*; following tumour cells injection, animal weight and tumour growth were monitored once a week. Animals were killed at the end of experiment by cervical disruption; as no signs of suffering appeared during the experiment (submitted attitude, weight loss, prostration, vocalisation), no animal has to be killed before the end of experiment.

Treatment

Mice bearing well-established CAL33 tumours (mean tumour volume/treatment group $\sim 250 \text{ mm}^3$) were treated each week with vehicle alone (controls), AZD2171 (2.5 mg kg^{-1} every day 0.2 ml p.o.), gefitinib ($50 \text{ mg kg}^{-1} \text{ day}^{-1}$, 5 days per week, 0.2 ml p.o.) and RT (6 Gy day^{-1} , 3 days per week, 2 h after drugs administration) for 2 weeks. When coadministered, AZD2171 and gefitinib were given simultaneously. The dose of gefitinib and AZD2171 were chosen according to preliminary experiments so that each drug given alone exerts only partial effects on tumour growth.

Radiotherapy was performed with γ -rays on tumour only, using a ^{60}Co unit, the animals being maintained and biologically isolated from ambient air during RT with a straitjacket made of Saran wrap film.

The effect of AZD2171 and gefitinib alone or in combination with RT on tumour growth was evaluated. The effects of the treatments were calculated, as described previously (Prewett *et al*, 2002). Evaluation of the effects on tumour growth consisted in measuring, for all groups, the mean tumour volume at the end of

the observation period for the controls (day 30), when tumours in this group reached the average volume of 2500 mm^3 (maximal ethically acceptable volume). Fractional tumour volume (FTV) for each treatment group was calculated as the ratio between the mean tumour volumes of treated and untreated animals. This was performed for treatment a (FTVa), for treatment b (FTVb) and for treatment a + b (FTV a + b). The expected FTV for the a + b combination was defined as FTVa observed \times FTVb observed. The ratio FTVa + b expected/FTVa + b observed was the combination ratio (CR). If $\text{CR} > 1$, there are supra-additive effects and if $\text{CR} < 1$ infra-additive ones. Strictly additive effects were observed if $\text{CR} = 1$. Another way to compare treatment effects was to calculate the time necessary for the tumours to reach a volume of 1000 mm^3 (log-rank test).

Tumour analysis

Tumour length and width was measured weekly using a calliper rule. Tumour volume was calculated as $\pi/6 \times \text{length} \times \text{width}^2$ until animal killing. Animals were killed by spinal cord dislocation and tumours were subsequently removed surgically. Half of the tumour was directly frozen in liquid nitrogen for protein analysis and the other half fixed in paraformaldehyde overnight for analysis of the microvessel marker, von Willebrand factor (vWF; Dako, polyclonal antibody ref.: A0082), and the proliferation marker, Ki67 (Dako, Trappes, France, monoclonal antibody ref.: M7240, MIB-1), using immunohistochemistry after tumour tissue from the experimental groups was assembled into microtissue arrays (Simon *et al*, 2004). The analysis of vWF concerned both vessel loss and reduction in vessel area. The analysis of Ki67 took into account both the intensity of labelling and proportion of labelled cells.

The final score was the result of the examination of three fields per tumour, and between four and five tumours were investigated for each treatment group. One hundred $\times 10$ magnification images of regions of high vascular density within the tumour were analysed in order to quantify tumour angiogenesis (vWF). Staining intensities were scored as: 1 = slight; 2 = medium; and 3 = strong. A subgroup ($n = 5$) was killed on day 23 (the last day of treatment) and tumours collected, while the remaining animals ($n = 5$) were killed in each group, once animals in the group reached a tumour volume of 2500 mm^3 . Approximately, these times were reached on day 30 for control and gefitinib, on day 37 for AZD2171 and AZD2171 + gefitinib, on day 50 for RT, and on day 60 for AZD2171 + gefitinib + RT treatment groups.

Frozen tumours were pulverised in a liquid nitrogen-cooled Thermovac pulveriser. The resulting powders were homogenised in 10 volumes of a 10 mM Tris-HCl buffer pH 7.4, containing 1 mM EDTA, 0.5 mM dithiothreitol, 10 mM sodium molybdate, phosphatase inhibitor cocktail 2 with a dilution 1/100 and protease inhibition cocktail 2 with a dilution 1/100, both from Sigma (Saint Quentin Fallavier, France). The homogenates were centrifuged for 1 h at $105\,000 \text{ g}$ ($+4^\circ\text{C}$) and the supernatants (cytosols) were used for protein determination by immunoblotting. Total protein content was measured using the bicinchoninic acid assay.

Human VEGF secreted by CAL33 xenografted tumours was determined in tumour cytosol by ELISA using DVE00 (Quantikine, R&D systems, Lille, France). Epidermal growth factor receptor had been previously determined by the ^{125}I -EGF-binding method followed by Scatchard-plot analysis (Dassonville *et al*, 1993).

The EGFR and VEGFR signalling pathway markers phospho-ERK1/2, PTEN and phospho-AKT, the apoptosis-related markers Bax/Bcl2 ratio, the proliferation-related marker p27 and the DNA repair-related marker ERCC1 were determined by Western blot (detailed technical conditions are summarised in Table 1).

Two sets of tumours have been analysed with different purposes. One set (five animals per treatment group) was collected at the end of treatment period (day 23) in order to compare the effects of the different treatments on a panel of molecular markers. The other set

Table 1 Technical conditions of Western blot analyses

Antibody	% Acrylamide	Dilution	Source	Supplier
PTEN	12	1/1000 TBS 5% milk	Mouse	Pharmingen
ERCC1	12	1/500 TBS 5% milk	Mouse	Neomarkers
Phospho-AKT	12	1/1000 TBS 5% BSA	Rabbit	Ozyme
p27	12	1/1000 TBS 5% milk	Rabbit	Pharmingen
Bax	12	1/1000 TBS 5% milk	Rabbit	Upstate
Bcl2	12	1/1000 TBS 5% milk	Rabbit	Pharmingen
Phospho-ERK1/2	12	1/5000 TBS 5% milk	Mouse	Sigma

BSA = bovine serum albumin; TBS = tris-buffered saline.

of tumour-bearing animals was maintained to assess tumour regrowth after the end of treatment period. Animals were killed when mean tumour volume of each treatment group reached 2500 mm³. These tumours were used to detect what molecular parameters could be modified in these tumours escaping to treatments. The first set of tumours was analysed for vWF (endothelial cell-specific marker) and Ki67 (proliferative capacity of tumour cells) by immunohistochemistry, for the EGFR and VEGFR signalling pathway markers such as phospho-ERK1/2 and phospho-AKT, for the apoptosis-related markers Bax/Bcl2 ratio, the proliferation-related markers p21 and p27 and the DNA repair-related marker ERCC1 (essentially on tumour cells as they largely outnumber endothelial cells in tumours) by Western blot normalised by Raf as a loading control, and VEGF (human, secreted by tumour cells only) by ELISA.

The second set of tumours was analysed for phospho-ERK1/2, phospho-AKT and PTEN expression by Western blot also normalised by Raf as a loading control.

The bands in the Western blots were quantified using the Chemi Doc imager from Bio-Rad, Marnes-La-Coquette, France.

Statistical analyses

Comparison of tumour growth between different treatment groups was performed by Kaplan–Meier-type analysis with the log-rank statistical test. The effects of treatment on vWF and Ki67 were evaluated using the non-parametric ANOVA (Kruskal–Wallis test). The differences between treatment groups for molecular factors (VEGF, phospho-ERK1/2, PTEN, phospho-AKT, Bax/Bcl2, ERCC1) were examined using the Mann–Whitney test.

RESULTS

Effects of AZD2171 in combination with gefitinib and radiation on tumour growth

The effect of the different treatments on tumour growth is shown in Figure 1. Tumour growth was inhibited by AZD2171 or gefitinib given alone. These antitumour effects were rapidly established but disappeared after treatment cessation. In contrast, the antitumour effects of RT were only seen after 1 week of treatment and persisted over a longer period. Combination treatment with AZD2171 + gefitinib produced a greater inhibition of tumour growth than either treatment alone and led to a growth arrest. Tumour regrowth occurred after the end of treatment in all groups with either drugs alone or when combining AZD2171 with gefitinib.

We then considered the AZD2171–gefitinib combination as a single-drug treatment and examined the effects of its combination associated with RT. The triple combination (AZD2171 + gefitinib + RT) produced a greater antitumour effect than either RT or the AZD2171–gefitinib combination. In comparison to RT or to the AZD2171–gefitinib combination, the triple combination prolonged the antitumour effects after treatment discontinued.

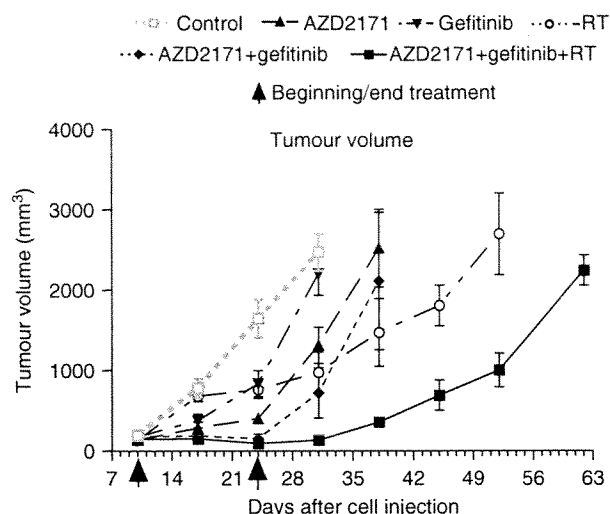


Figure 1 Effects of AZD2171 (2.5 mg kg⁻¹, every day during 2 weeks), gefitinib (50 mg kg⁻¹, 5 days per week during 2 weeks), RT (6 Gy, three times per week, every 2 days during 2 weeks, 2 h after drugs) or a combination on growth of CAL33 head and neck tumours (mean tumour volume \pm s.d., $n = 10$ per treatment condition until day 24, then $n = 5$ per treatment group except for RT where $n = 4$).

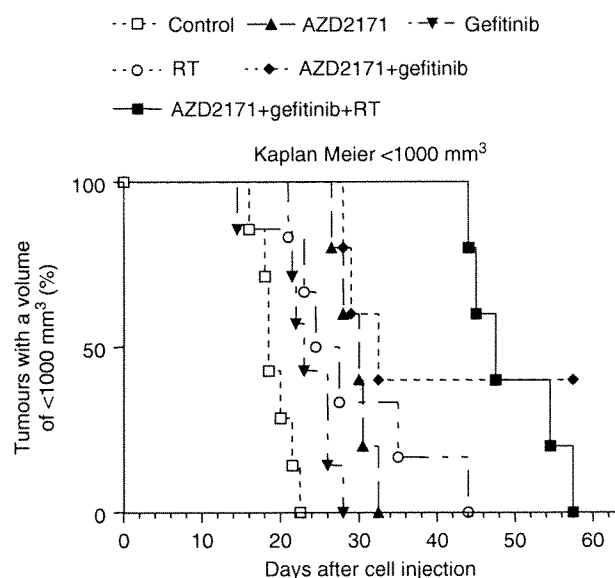


Figure 2 Respective time delay to reach a tumour volume of 1000 mm³ for the different treatment groups (seven mice for control and gefitinib, six mice for RT and five mice for all other treatment groups). Of note, two tumours never reached the 1000 mm³ volume (these tumours were purely necrotic but still present at the end of the experiment).

Following the treatment period with the triple combination, growth arrest lasted for 1 week, followed during 3 weeks by a growth paralleling that of the RT-treated tumours and by a rapid growth during the last 10 days.

Antitumour effects for AZD2171 + gefitinib in combination were supra-additive (CR = 1.6) as were those for the triple combination of both drugs administered with RT (CR = 2). These CR values (> 1) are consistent with tumour growth inhibition observed with each regimen.

The median time to reach a tumour volume of 1000 mm³ was significantly increased for AZD2171 or gefitinib alone compared

with the control ($P = 0.001$). There was also a significant difference between the combination of AZD2171 + gefitinib and either drug alone ($P = 0.006$) and between the triple combination and AZD2171 + gefitinib ($P = 0.0001$) or RT alone ($P < 0.0001$; Figure 2).

Body weights were measured as surrogate markers of treatment tolerance with a slight body weight loss in all treatment groups compared to the control group, thus suggesting that treatments were well tolerated (Figure 3).

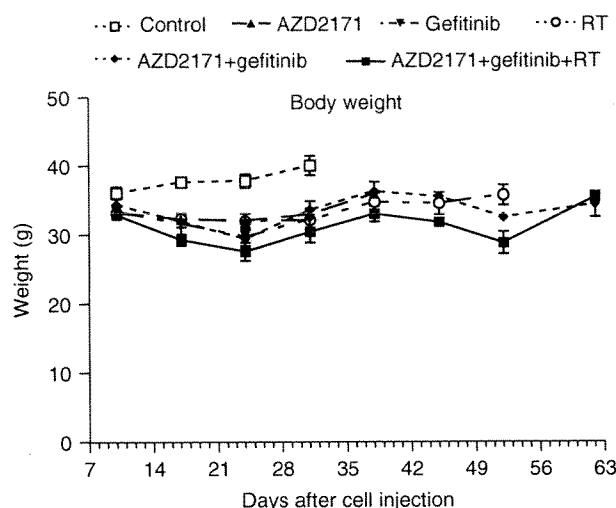


Figure 3 Profile of body weight vs time among the different treatment groups (mean weight \pm s.d., $n = 10$ per treatment condition until day 24, $n = 5$ thereafter).

Effects of treatments on molecular parameters

In the first set of tumours (collected at the end of treatment period), the intensity of vWF staining was slightly reduced by each agent given alone, and markedly diminished by the double or triple combination ($P < 0.0001$ for triple association vs control; Figure 4).

The triple combination almost completely abolished proliferation in tumour cells as shown by the decrease in Ki67 labelling intensity ($P < 0.001$ for triple association vs control; Figure 5). The effects on apoptosis-related factors Bax/Bcl2 ratio were inconsistent (data not shown). No significant effect on phospho-ERK1/2 and on phospho-AKT was observed with any of the treatment groups (data not shown).

The marked RT-induced enhancement in ERCC1 expression was totally abolished by the triple combination ($P = 0.04$ for the triple combination vs RT; Figure 6). This result could help to explain the supra-additive antitumour effect produced by this combination.

Compared with VEGF levels in the control tumours, gefitinib alone tended to decrease VEGF concentration ($P = 0.157$), whereas AZD2171 alone enhanced levels of VEGF in the tumour ($P = 0.02$ vs control). The combination AZD2171 + gefitinib as well as RT alone had no effect on VEGF levels, whereas the triple combination was associated with an increase in VEGF (Figure 7).

In addition to measurements of tumour factors at the end of treatment, there were molecular investigations performed in the second set of tumours (obtained at the end of follow-up when tumours had regrown). All tumours were collected at a mean volume of 2500 mm^3 . There was a variable diminution of phospho-ERK1/2 expression as compared to the control for all treatment conditions (data not shown). All the treatment groups showed an increase in phospho-AKT expression with the triple association showing the largest increase ($P < 0.0001$; Figure 8). All treatment groups showed a marked decrease in PTEN expression, the phosphorylase, which negatively controls the PI3-Akt pathway (reaching statistical significance for RT and gefitinib + AZD2171 combination only, $P = 0.04$ and 0.03 , respectively; Figure 9). These

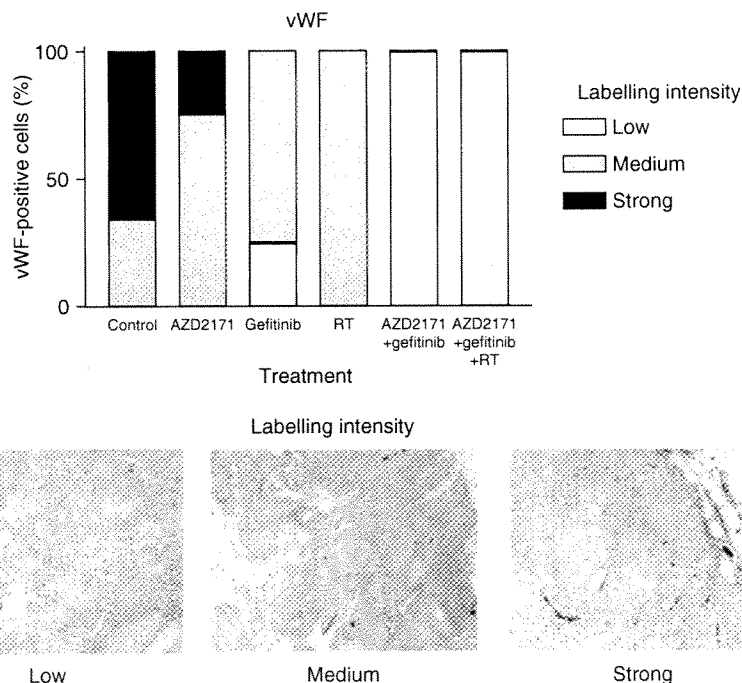


Figure 4 Impact of the different treatments on day 24 at the end of the treatment period for the different treatments on vWF (endothelial cell marker), four microscope fields observed on four tumours (conditions AZD2171, gefitinib, AZD2171-gefitinib), five tumours (conditions RT, AZD2171-gefitinib-RT), six tumours (control). Magnification $\times 20$ for labelling intensity.

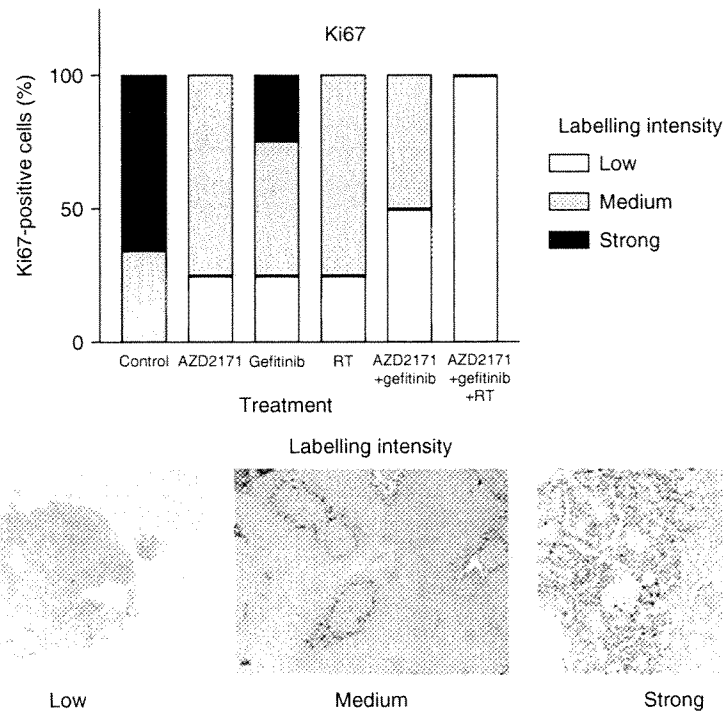


Figure 5 Impact of the different treatments on Ki67 staining (proliferation marker) on day 24 at the end of the treatment period for the different treatments (four microscope fields observed) on four tumours (conditions AZD2171, gefitinib, AZD2171–gefitinib), five tumours (conditions RT, AZD2171–gefitinib–RT), six tumours (control). Magnification for labelling intensity: $\times 4$ for low and $\times 20$ for medium and strong.

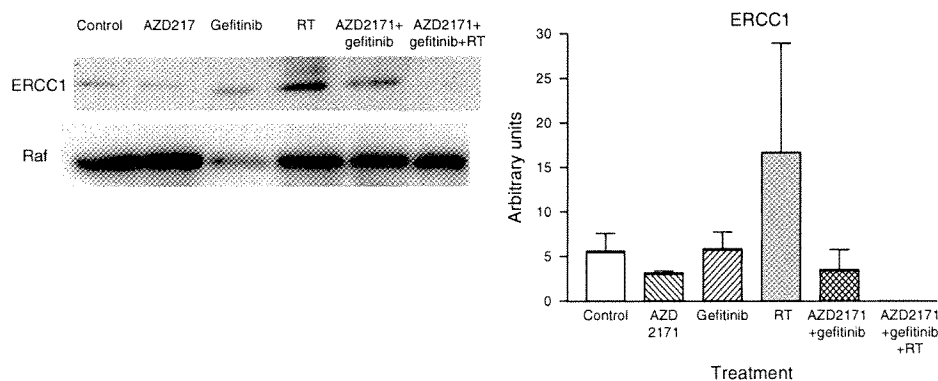


Figure 6 Effect of the different treatments on the expression of ERCC1 on day 24 at the end of the treatment period for the different treatments (mean expression \pm s.d.), three tumours for control and AZD2171–gefitinib–RT and four tumours for all other treatment conditions. A typical example of Western blot analysis is given. The results were normalised vs Raf taken as a loading control.

results corroborate the effects observed on phospho-AKT. In addition, all treatments except gefitinib alone showed a marked decrease in the expression of the cell-cycle regulating protein p27 ($P = 0.001$; Figure 10). Results with p21 were less consistent (data not shown).

DISCUSSION

Several preclinical studies have examined the antitumour activity of inhibitors of EGFR and antiangiogenic agents in combination and have demonstrated at least additive, if not synergistic, effects (Ciardiello *et al*, 2000; Jung *et al*, 2002; Herbst *et al*, 2003). These encouraging data have led to the initiation of clinical studies in lung cancer, evaluating the combination of erlotinib, an EGFR

tyrosine kinase inhibitor, with bevacizumab, an anti-VEGF antibody (Herbst *et al*, 2005). Previous experimental studies showed potential beneficial antitumour effects when combining antiangiogenic agents with RT (Wachsberger *et al*, 2005; Nieder *et al*, 2006), resulting in at least additive effects on tumour growth delay despite different radiation schedules, drugs and doses and combination regimens. Clinical research in this field is ongoing but additional preclinical studies are needed to further evaluate drug combinations, including the targeting of EGFR and VEGF signalling pathways in association with RT. The present study was designed in a similar way to work published by Raben *et al* (2004) who combined gefitinib with vandetanib (a vascular-targeting agent) and RT. The authors reported that the triple association induced the greatest effect on tumour growth and angiogenesis.

The present data indicate that the double combination, the concomitant application of the VEGF signalling inhibitor, AZD2171, with gefitinib produced more than additive effects with a CR value of 1.6, and that the triple combination, the concomitant

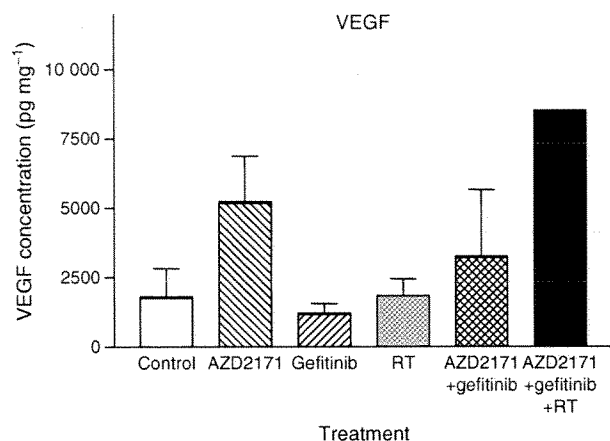


Figure 7 Human VEGF tumour concentration (pg mg⁻¹ protein) on day 24 at the end of the treatment period for the different treatments (mean concentration \pm s.d.), three tumours for controls and AZD2171, four tumours for conditions with gefitinib, RT and AZD2171–gefitinib; the triple combination could be analysed on one tumour only, due to the very small size of the tumours reflecting the efficiency of the combined treatment.

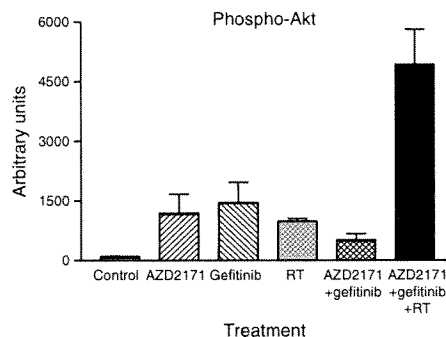
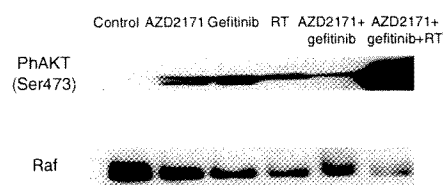


Figure 8 Phospho-AKT expression for tumours in regrowth following different treatments. Tumours collected when mean tumour volume in each group reached 2500 mm³ (mean \pm s.d., three tumours for each treatment condition). A typical example of Western blot analysis is given. The results were normalised vs Raf taken as a loading control.

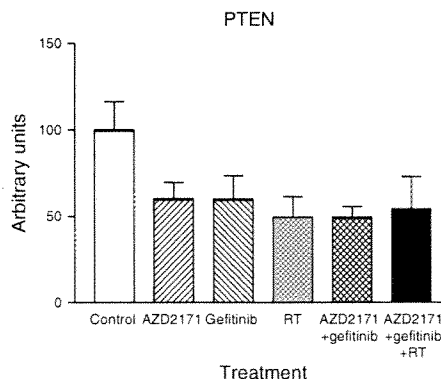
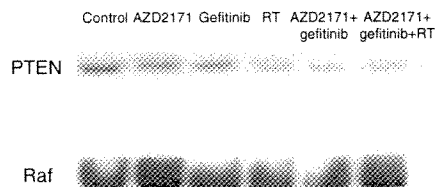


Figure 9 PTEN expression for tumours in regrowth following the different treatments. Tumours collected at the end of follow-up when mean tumour volume in each group reached 2500 mm³ (mean \pm s.d., three tumours for each treatment condition). A typical example of Western blot analysis is given. The results were normalised vs Raf taken as a loading control.

application of the VEGF signalling inhibitor, AZD2171–gefitinib association with RT, produced the highest supra-additive anti-tumour effects with a CR value of 2. Importantly, this triple combination maintained a longer growth delay in comparison either to AZD2171–gefitinib double combination or to RT. The exploration of several tumour markers sustained the antitumour effects, which were observed. Among them, it was interesting to note that the antitumour efficacy of the triple combination could be attributed both to a direct impact on tumour cell proliferation (Ki67 with a marked decrease in labelling) and on the endothelial cell network of the tumour (vWF staining). A previous experimental study by our group was performed on an immortalised microvascular endothelial cell line of human origin (Bozec *et al*, 2005). This cell line was exposed to a combination, including gefitinib, with ZM317450 a tyrosine kinase inhibitor against VEGFR-2 and RT. A marked synergistic interaction for cytotoxic effect was found with this triple combination only. These data concur well with the present results showing a decrease of the number of endothelial cells, as indicated by vWF staining, by combining AZD2171 with gefitinib and RT compared to untreated controls and either monotherapy. It was not within the scope of the present study to examine the effects of the respective associations between single drugs and RT. The AZD2171–gefitinib combination was taken as a whole when combined with RT.

Blood plasma levels of VEGF are significantly increased by VEGFR-2 blockade in mice and were proposed as a surrogate marker for VEGFR-2 targeted therapy in the clinical situation (Jain *et al*, 2006). Sunitinib, a tyrosine kinase inhibitor of VEGFRs, was shown to increase plasma levels of VEGF in treated patients (Faivre

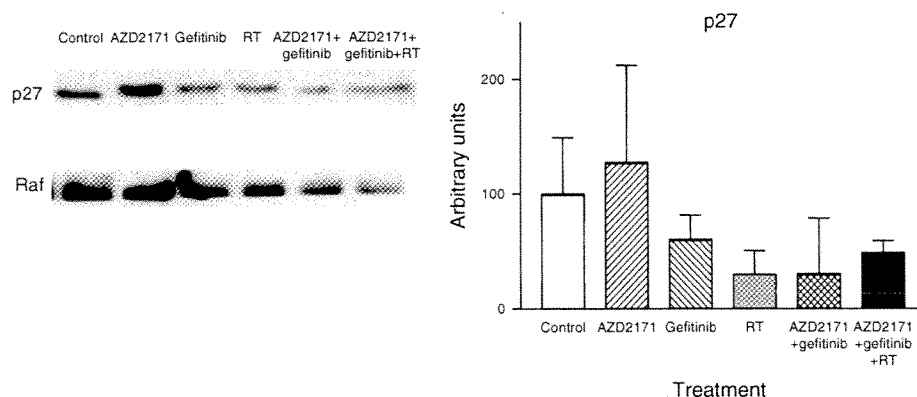


Figure 10 p27 expression for tumours in regrowth following the different treatments. Tumours collected when mean tumour volume in each group reached 2500 mm³ (mean \pm s.d., three tumours for each treatment condition). A typical example of Western blot analysis is given. The results were normalised vs Raf taken as a loading control.

et al, 2006a). The present data indicate that AZD2171, a highly potent VEGFR-2 inhibitor, was able to increase VEGF tumour expression. The strict tumour origin of the measured VEGF was based on the fact that the measured VEGF was of human origin. This observation confirms that changes in VEGF levels under treatment may be the reflect of modifications occurring in tumour VEGF expression.

One of the molecular explanations for the supra-additive effects on tumour growth presently observed may lie in the changes in tumour expression of the DNA repair protein ERCC1. As expected, ERCC1 expression was markedly enhanced by RT treatment alone. When combined with AZD2171 and gefitinib, this RT-dependent induction of ERCC1 was totally abrogated (Figure 6).

PTEN inactivation and constitutive activation of AKT are well-defined genetic alterations in the initiation and progression of tumours (Vivanco and Sawyers, 2002). AKT is a crucial survival kinase, and interfering with AKT expression is an attractive

strategy to control tumour progression (Goswami *et al*, 2006). Interestingly, the present data show that tumour regrowth was accompanied by marked changes in tumour expression of PTEN and phospho-AKT. PTEN was found to be significantly decreased. Conversely, phospho-AKT expression was enhanced as compared to controls. This was particularly true in tumours having shown the highest response under treatment (with the triple combination), AKT is one of the main mTOR-related messengers (Smolewski, 2006) and the current development of mTOR inhibitors as anticancer drugs is very promising (Faivre *et al*, 2006b). It is suggested that to add mTOR targeting as an additional sequence of treatment following the presently studied combination could maintain prolonged antitumour effects over time. Overall, the present data highlight the interesting antitumour effects of the triple combination with gefitinib, AZD2171 and RT. This strategy could serve as a basis for future innovative clinical trials.

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